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IL-6 SUPPORTS THE GENERATION OF HUMAN LONG-LIVED PLASMA CELLS IN COMBINATION WITH EITHER APRIL OR STROMAL CELL SOLUBLE FACTORS

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Running title: Obtaining human long-lived plasma cells *in vitro*

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ABSTRACT

The recent understanding of plasma cell (PC) biology has been obtained from murine models mainly. The current concept is that plasmablasts home to the BM and further differentiate into long-lived PCs (LLPCs). These LLPCs survive for months in contact with a complex niche comprising stromal cells (SCs) and hematopoietic cells both producing recruitment and survival factors. Using a multi-step culture system, we show here the possibility to differentiate human memory B cells into LLPCs surviving for at least 4 months *in vitro* and producing immunoglobulins continuously. A remarkable feature is that IL-6 is mandatory to generate LLPCs *in vitro* together with either APRIL or soluble factors produced by SCs, unrelated to APRIL/BAFF, SDF-1, or IGF-1. These LLPCs are out of the cell cycle, express highly PC transcription factors and surface markers.

This model shows a remarkable robustness of human LLPCs, which can survive and produce highly immunoglobulins for months *in vitro* without contact with niche cells, providing the presence of a minimal cocktail of growth factors and nutrients. This model should be useful to understand further normal PC biology and its deregulation in premalignant or malignant PC disorders.

Key words: Plasma cell; B cell; differentiation; microarray

INTRODUCTION

Mature memory plasma cells (PCs), termed long lived plasma cells (LLPCs), are located in the bone marrow (BM) or mucosa and may survive for years, insuring long-term immune memory.[1] LLPCs are rare cells (0.25 % of BM cells)[2,3] making their study difficult in humans. Recent knowledge about their generation and maintenance was obtained with murine models mainly. After selection of antigen (Ag) specific B cells in the germinal center, involving mutations in immunoglobulin (Ig) variable genes and isotype switching, centrocytes differentiate into memory B cells (MBCs) or plasmablasts (PBs). These PBs migrate to medullary cords, exit into the lymph through a sphingosine phosphate gradient and get to the peripheral blood.[4] Circulating PBs have to find a specific niche in the BM or mucosa that will provide them with the factors to survive and fully differentiate. The restricted number of suitable PC niches is supposed to be the main limiting factor explaining LLPC rarity.[4] In mice, Tokoyoda *et al*. have reported the PC niche to be a $VCAM1^+$ $SDF\text{-}1^+$ stromal cell that could be shared by hematopoietic progenitors and pre-pro B cells.[5] Several hematopoietic cell subsets have been described to be involve in PC retention, maturation, and maintenance in the BM, including macrophages, eosinophils, and megakaryocytes.[6] At least 3 growth factors and chemokines, produced by the exquisite PC niche, are recognized to control PC survival: SDF-1, APRIL/BAFF, and IL-6.[4] SDF-1, produced by SCs is essential to recruit mouse PBs into the BM.[5,7] BAFF and APRIL are produced by hematopoietic cells and are critical to support PC survival.[6] BAFF binds to 3 receptors, BAFF receptor, BCMA and TACI, whereas APRIL binds to BCMA and TACI *in vivo*.[8] Although both APRIL and BAFF can support murine LLPC survival,[9] APRIL appears to be more efficient than BAFF to promote LLPC survival, at least in mice. First, APRIL binds to BCMA with a higher

affinity than BAFF,[8] and BCMA is highly expressed in PCs.[10] Secondly, APRIL and its receptor TACI bind to heparan sulfate chains, in particular to the proteoglycan syndecan-1,[11,12] which is a hallmark of mature PCs.[13] Thirdly, the long-term survival of transferred LLPCs is impaired in APRIL$^{-/-}$ mice, but unaffected in BAFF$^{-/-}$ ones.[14] IL-6, produced by SCs or dendritic cells, is essential for the generation of PBs, and its role in supporting LLPC survival is controversial in mice. The generation of Ag-specific PCs is dramatically decreased in IL-6$^{-/-}$ mice[15] and the ability of BM SCs to support the survival of purified BM LLPCs *in vitro* is inhibited by an anti-IL-6 monoclonal antibody (mAb) or lost using IL-6$^{-/-}$ mice SCs.[16,17] Similarly, the survival of LLPCs in the culture of intestine biopsies is impaired by an anti-IL-6 mAb or an APRIL/BAFF inhibitor.[18] But the persistence of LLPCs transferred into IL-6$^{-/-}$ mice is unaffected.[14]

In humans, the factors that promote the differentiation of PBs into LLPCs are poorly identified *in vivo*, due to the rarity of bone marrow PCs (BMPCs) and the ethical difficulty to harvest the BM. The majority of the studies dealing with PC survival and growth were done with malignant PCs.[19,20] Using *in vitro* models of differentiation of B cells into PCs, we and others have shown that *in vitro* generated PCs are early PCs with a phenotype close to that of circulating PBs and PCs in healthy individuals.[10,21,22] These *in vitro* generated PCs expressed highly CD38 and CD31 and lack B cell antigens (CD20, CD22, CD24) except CD19. They express CD138 but at lower level than BMPCs, they express CD62L unlike BMPCs, and fail to express CD9, VCAM1, and CCR2.[10] Recent studies have proposed a role for osteoclasts[23] or SCs to support human PC survival *in vitro*.[24] In this last study, blood B cells could be differentiated into Ig-secreting LLPCs in contact with soluble factors produced by a mouse SC line. However, the molecular mechanisms of this supportive activity remain unknown.[24]

In the current study, we show that IL-6 is mandatory for the *in vitro* survival of LLPCs in combination with either APRIL or BAFF or APRIL/BAFF-unrelated SC soluble factors. These LLPCs are non-cell cycling, survive for months *in vitro*, while producing Igs continuously.

MATERIALS AND METHODS

Reagents

Human recombinant IL-2, TACI-Fc, and APRIL were purchased from R&D Systems (Minneapolis, MN), IFN-α (IntronA) from Merck Canada Inc. (Kirckland, Canada), IL-6 and IL-15 from AbCys SA (Paris, France), BAFF, SDF-1α, IGF-1, and IL-10, from Peprotech (Rocky Hill, NJ, USA), the B-E8 anti-IL-6 mouse mAb from Diaclone (Besançon, France), the SDF-1 inhibitor AMD3100 from Sigma (Sigma-Aldrich, St Louis, MO), the IGF-1R inhibitor from Novartis Pharma (Basel, Switzerland), and IKK16, a selective inhibitor targeting both IKK1 and IKK2, from R&D Systems.

Cell samples

Peripheral blood cells from healthy volunteers were purchased from the French Blood Center (Toulouse, France) and $CD19^+CD27^+$ MBCs purified (≥ 95% purity) as described.[10] When indicated, D10 early PCs ($CD20^-CD138^+$) were FACS-sorted using FITC-conjugated anti-CD20 mAb and PE- or APC-conjugated anti-CD138 mAb. The purity of FACS-sorted cell populations was ≥ 95% as assayed by cytometry. Resto-6 stromal cells were used as a source of SCs. These SCs were obtained from a 15%/25% Percoll interface of dissociated human tonsil cells as previously described.[25] Plastic-adherent cells were selected and expanded in RPMI 1640 culture medium and 10% fetal calf serum (FCS) yielding to the Resto-6 SCs after 8 passages. Resto-6 SCs express usual mesenchymal stromal cell markers (CD90, CD73, and CD105) and can acquire properties of fibroblastic reticular cells (FRC) including expression of high levels of adhesion molecules and gp38/podoplanin, production of a dense meshwork of transglutaminase, and production of inflammatory and lymphoid chemokines upon stimulation by TNFα and Lymphotoxin-α1β2.[25] Resto-6 SCs support efficiently the growth and survival of

normal B and T cells and of malignant lymphoma B cells, in particular after FRC-commitment. They were used between passages 8 and 15.

Cell cultures

PCs were generated through a four-step culture. All cultures were performed in Iscove's modified Dulbecco medium (IMDM, Invitrogen) and 10% FCS. In step 1, purified peripheral blood MBCs (1.5 x 10^5/ml) were activated for 4 days by CpG oligodeoxynucleotide and CD40 ligand (sCD40L) - 10 µg/ml of phosphorothioate CpG oligodeoxynucleotide 2006 (Sigma), 50 ng/ml histidine tagged sCD40L, and anti-poly-histidine mAb (5 µg/ml), (R&D Systems) - with IL-2 (20 U/ml), IL-10 (50 ng/ml) and IL-15 (10 ng/ml) in 6 well culture plates. In step 2, PBs were generated by removing CpG oligonucleotides and sCD40L and changing the cytokine cocktail (IL-2, 20 U/ml, IL-6, 50 ng/ml, IL-10, 50 ng/ml and IL-15, 10 ng/ml). In step 3, PBs were differentiated into early PCs adding IL-6 (50 ng/ml), IL-15 (10 ng/ml) and IFN-α500 U/ml) for 3 days. In step 4, early PCs were differentiated into LLPCs using either coculture with SCs, transwell culture, or a cytokine cocktail, and the cultures maintained for months. Confluent monolayers of SCs were generated in 6-, 24-, or 48-well flat-bottom culture plates and PCs were then added onto the monolayers together with various cytokines. These cocultures of PCs and SCs could be maintained for months with the same SC monolayer, adding fresh culture medium and growth factors once by week. Cocultures of PCs and SCs without cell contact were done seeding SCs in the lower chamber of 6-well transwells and PCs in the upper compartment, both compartments being separated by a 0.4 µm-polycarbonate membrane (Corning, New-York, NY). SC-conditioned medium (SC-CM) was obtained by culturing confluent monolayers of SCs for 5 days with culture medium. The culture supernatant was 0.2 µM filtered and frozen and 50% of SC-CM was added to PC

cultures, and renewed every week. Finally, LLPCs were also obtained adding IL-6 (10 ng/ml) and either APRIL (200 ng/ml) or BAFF (200 ng/ml).

Assay for cell viability and cell growth

Cell concentration and viability were assessed using trypan blue dye exclusion test. The number of metabolic active cells was also determined using intracellular ATP quantitation with a Cell Titer Glo Luminescent Assay (Promega Corporation, Madison, WI).

Cell cycle analysis, immunophenotypic analysis, and cytology

The cell cycle was assessed using DAPI staining (Sigma-Aldrich) and cells in the S phase using incubation with bromodeoxyuridine (BrdU) for 1 hour and labelling with an anti-BrdU antibody (APC BrdU flow kit, BD Biosciences) according to manufacturer's instructions. Cells were stained with a combination of 4 to 7 mAbs conjugated to different fluorochromes. The Cytofix/Cytoperm kit (BD Biosciences) was used for intracellular staining of IgM, IgA, IgG or Ki67 antigen.[10] Flow cytometry analysis was performed with a FACSAria cytometer using FACSDiva 6.1 (Becton Dickinson, San Jose, CA) and with a Cyan ADP cytometer driven by the Summit software (Beckman Coulter). Kaluza software (Beckman Coulter) was used for data analysis. The fluorescence intensity of the cell populations was quantified using the stain index (SI) formula: [mean fluorescence intensity (MFI) obtained from a given mAb minus MFI obtained with a control mAb]/[2 times the standard deviation of the MFI obtained with the same control mAb].[10] Cytospin smears of cell-sorted $CD20^-$ $CD138^+$ D30 PCs were stained with May-Grünwald-Giemsa.

Analysis of Ig secretion

ELISA. Flow cytometry sorted PCs were cultured at 10^6 cells/ml for 24 hours and culture supernatants harvested. IgM, IgA, or IgG concentrations were assessed by

ELISA using human IgM, IgA, and IgG ELISA kits from Bethyl Laboratories (Montgomery, TX), according to the manufacturer's recommendations.

ELISPOT. The number of IgM, IgA, or IgG secreting PCs was evaluated with the ELISPOT assay,[26] seeding 500 PCs by well in ELISPOT plates and culturing cells for 18 hours. The number and size of IgM, IgA, and IgG elispots were assessed using the Biosys Bioreader 5000 apparatus (Biosys, Miami, FL).

Microarray hybridization and bioinformatics analysis

RNA was extracted and hybridized to human genome U133 Plus 2.0 GeneChip microarrays, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Gene expression data are deposited in the ArrayExpress public database (http://www.ebi.ac.uk/microarray-as/ae/). The accession numbers are E-MEXP-3034 for prePBs, E-MEXP-2360 for PBs and BMPCs, E-MEXP-3945 for day 10 (D10) early PCs and D30 PCs, and E-MTAB-2118 for Resto-6 SCs. Gene expression data were analyzed with our bioinformatics platforms (RAGE, http://rage.montp.inserm.fr/)[27] and Amazonia (http://amazonia.transcriptome.eu/).[28] Genes differentially expressed between cell populations were determined with the SAM statistical microarray analysis software.[29] The clustering was performed and visualized with the Cluster and TreeView softwares.[30] Gene annotation and networks were generated with the Reactome Functional Interaction Cytoscape plugin (http://www.cytoscape.org/).

Statistical analysis

Statistical comparisons were made with the non-parametric Mann-Whitney test, unpaired or paired Student's *t*-test using SPSS software. *P*-values $\leq .05$ were considered as significant.

RESULTS

IL-6 in combination with APRIL or BAFF or APRIL/BAFF-unrelated stromal-cell soluble factors supports the generation and survival of PCs *in vitro*

Starting from MBCs, early PCs can be generated within 10 days using a 3-step culture. These early PCs could survive poorly in presence of IL-6 (Figure 1A). Adding human tonsil SCs could promote PC survival with 28% surviving CD138 PCs at day 30 (Figure 1A). In the presence of SCs, the CD138 fluorescence staining index at day 14 was increased 3 fold (*P* = .006) compared to day 14 PCs generated with IL-6 alone (Figure 1B and 1C). It progressively increased 2 fold from day 14 to day 30 (*P = .032)* and then was stable up to day 60 (Figures 1B and 1C). Coculturing early PCs separated from SCs in transwell culture plates promoted PC survival at the same extent as coculture of PCs with SCs at day 14, 17, 24 and 30, showing a contact between PCs and SCs is not mandatory (Figure 2A). In agreement, coculture supernatant of PCs with SCs (PC/SC-CM) or of SCs alone (SC-CM) also promoted PC survival at day 30 (Figure 2A). Various growth recombinant factors - APRIL, BAFF, SDF-1, IGF-1 - known to sustain malignant PC survival[19] did not induce PC survival when used alone (Figure 2B). IL-6 alone supported PC survival but at a 76% lower level than that induced by the SC-CM (*P* = .003). Adding APRIL together with IL-6 increased significantly 6.5 fold (*P* = .02) the IL-6 PC survival activity (Figure 2B). Adding BAFF also increased the PC survival induced by IL-6 (*P* = .02) and SDF-1 and/or IGF-1 did not increase IL-6 activity (Figure 2B). The PC survival induced by the SC-CM was fully abrogated by an anti-IL-6 mAb showing the critical role of IL-6 produced by SCs, but was unaffected by an APRIL/BAFF inhibitor, TACI-Fc (Figure 2C). The TACI-Fc APRIL/BAFF inhibitor fully blocked the ability of APRIL to increase 2.2 fold the PC generation induced by the SC-CM (Figure 2C). It also inhibited the

APRIL-induced growth of the XG1 myeloma cell line (supplementary Figure 1).[31] As APRIL/BAFF activate NF-κB pathways,[32] we investigated whether these pathways could be involved in PC generation by SCs. An inhibitor of both canonical and alternative NF-κB pathways (IKK16) did not affect the generation of PCs supported by SCs, whereas it impeded the additive effect of APRIL in PC generation promoted by SCs (Figure 3). Thus, SCs produce communication signals, which cooperate with IL-6 to promote PC survival and are not inhibited by a NF-κB inhibitor. This is in agreement with the low expression of NF-κB-induced genes[33] in PCs generated with SCs *in vitro* contrarily to BMPCs (supplementary Figure 2). These communication signals are not SDF-1 or IGF-1 since the SC-CM activity was unaffected by their specific inhibitors (Figure 2C). Adding these 3 inhibitors together (APRIL/BAFF, SDF-1 and IGF-1 inhibitors) yielded to an apparent decrease in SC-CM induced PC generation, which did not reach statistical significance (Figure 2C).

Of note, PCs could survive up to 120 days in these culture conditions adding fresh IL-6, APRIL and culture medium weekly and cultures were stopped after 120 days for convenience but not due to a decline in PC survival. These PCs were thus termed LLPCs. These results indicate that IL-6 is mandatory to induce the long-term survival of LLPCs in combination with either APRIL or BAFF or with APRIL/BAFF-unrelated soluble factors produced by SCs.

Characterization of D30 PCs

D30 PCs had a more mature PC phenotype than D10 early PCs with a progressive 7.4-fold increase in CD138 density (Figure 1C, $P \leq .001$), a 2.2-fold increase in CD54 staining index, a 1.5-fold increase in $CD9^+$ PCs, and a decrease in HLA-DR, CD45, CD62L, CCR10 expressing PCs and staining indexes ($P \leq .05$, Figure 4 and supplementary Figure 3). D30 PCs did not proliferate (0.04% cells incorporating

BrdU), whereas a low fraction of D10 early PCs was in the S phase of the cell cycle (4% BrdU+) (Figure 5A and 5B). D30 PCs displayed the cytology of mature PCs and produced cytoplasmic kappa or lambda Ig light chains (supplementary Figure 4). Day 60 PCs comprise IgG PCs only, with a quick disappearance of IgM PCs (no more detectable at day 30) and a progressive one of IgA PCs as assayed by FACS, ELISA and ELISPOT assays (Figures 6 A-F). Of note, the rate of IgG production was similar between day 30 and day 60 PCs (Figure 6F). The number of IgG and IgM producing cells and the Ig isotype produced were not significantly different starting from the same number of LLPCs generated with either SC coculture, IL-6+APRIL, SC-CM or APRIL+SC-CM (Figure 6G). There was a trend in increasing IgA secreting cells in the two experiments adding APRIL compared to the culture groups without APRIL ($P <$ .1). Combining data of these two experiments show APRIL increased 3.4 fold the number of IgA secreting cells (47 vs. 14 IgA secreting cells/500 PCs, $P <$ .001, Figure 6G).

B and PC transcription factors

D30 PCs had a higher expression of genes coding for IRF4 and BLIMP1 PC transcription factors than D10 early PCs and failed to express *PAX5* ($P \leq$ .02, Figure 7). D30 PCs expressed 3-fold more the spliced form of *XBP1* mRNA than D10 early PCs and 2-fold more the unspliced mRNA form than early PCs, resulting in an increased *XBP1s*/*XBP1u* mRNA ratio ($P \leq$ .009, Figure 7). Of note, *BCL6* gene expression in D30 PCs was low compared to that in B lymphocytes but significantly higher than that in early PCs ($P =$ .04, Figure 7).

Gene expression profile (GEP) of D30 PCs

In vitro generated D4 prePBs, D7 PBs, D10 early PCs, D30 PCs, and BMPCs purified from healthy individuals were profiled using Affymetrix U133 plus 2.0

microarrays. D30 PCs were generated using cocultures with SCs. The 5 populations are classified into 2 major clusters, a PC cluster comprising D10 early PCs, D30 PCs, and BMPCs and a PB cluster comprising prePBs and PBs (Figure 8). To look for genes indicator of LLPCs, we ran a SAM supervised analysis comparing D10 early PCs to D30PCs+BMPCs starting from the 5000 genes with the highest variance. 160 probe sets (141 unique genes) were overexpressed in D30PCs+BMPCs compared to D10 early PCs (Wilcoxon statistic, fold change ≥ 2, FDR ≤ 1%) and 490 (427 unique genes) in D10 early PCs versus D30PCs+BMPCs (supplementary Table 1). Genes coding for translation, focal adhesion, IL-6 signalling, and integrin signalling pathways were enriched in D30PC+BMPC genes (supplementary Table 2), and genes coding for DNA replication and mitosis in D10 early PCs (supplementary Table 3). The gene expression profiles of LLPCs harvested from the spleen of patients with primary immune thrombocytopenia treated with rituximab anti-CD20 mAb was recently documented and compared to that of PBs harvested from the spleen of untreated patients.[34] Similarly to the current *in vitro* generated LLPCs, LLPCs from these patients overexpress genes coding for PC transcription factors (*JUN, FOS, EGR1*), negative regulators of the cell cycle (*KLF4, KLF2, PPP1R15A*) and cell surface/cytokine receptors (*CD9, SDC1, FCRL5*). Conversely, similarly to *in vitro*-generated D10 early PCs, patients' PBs overexpress genes coding for positive cell cycle regulators (*CCND2, BUB1B, BUB1, TIMELESS, CENPF, MAD2L1, BIRC5, ZWINT, MKI67, MCM4, CCNB2*) and surface/cytokine receptors (*ITGB1, TNFSF10*) (supplementary Table 1). Comparing gene expression between BMPCs and D30 PCs, 198 unique genes were overexpressed in BMPCs and 555 in D30 PCs (SAM supervised analysis, Wilcoxon statistic, fold change ≥ 2, FDR ≤ 1%, supplementary Table 4). Genes coding for protein metabolism, translation, antigen processing and

305 presentation, and CXCR4 signalling were enriched in BMPC genes (supplementary
306 Table 5). Genes coding for glypican pathway, TGFβ receptor and Smad2/3
307 signalling, protein export and proteasome were enriched in D30 PC genes
308 (supplementary Table 6). GEP were done using D30 PCs generated with SCs and
309 IL-6. It could be of interest to investigate further whether adding APRIL together with
310 IL-6 or SC-CM could change the gene expression profiling, making it closer to that of
311 BMPCs.

DISCUSSION

This study shows i) the feasibility to generate human mature PCs *in vitro*, ii) the long-term survival of these PCs does not require a contact with niche cells, but only cell communication factors, in particular IL-6 and APRIL.

These PCs are called long-lived PCs because they are non-cycling PCs surviving and producing Igs for months *in vitro* as their counterpart *in vivo*.[1,35,36] In addition, they have a phenotype similar to that of LLPCs *in vivo*: high expression of CD138, increased expression of CD9, weak expression of CD62L, CD45 and HLA-DR compared to PBs and early PCs.[22,37] These *in vitro* generated LLPCs expressed *IRF4 and PRDM1* genes coding for PC transcription factors at a higher level than early PCs. Murine LLPCs also highly express Blimp1 compared to early PCs in the BM.[38] XBP1 is a master regulator of unfold protein response critical to protect PCs from stress induced by high Ig production.[39] *XBP1* mRNA has to be spliced to encode for an active protein and, in agreement, we found that LLPCs had an increased ratio of spliced to unspliced *XBP1* mRNAs compared to early PCs. LLPCs expressed weakly but significantly *BCL6* gene compared to early PCs. This deserves further study since Bcl6 is also inducible in malignant PCs in response to SC-derived factors, conferring a survival advantage on them.[2]

A second major finding is that the generation and survival of human LLPCs do not require a contact with niche cells *in vitro*, but can be obtained with 2 recombinant growth factors only, IL-6 and APRIL. APRIL can be replaced by BAFF, which activates the same receptors. In addition, APRIL can be replaced by APRIL/BAFF-unrelated soluble factors produced by SCs. Of note, an inhibitor of both the canonical and alternative NF-κB pathways did not affect the generation of LLPCs supported by SCs, whereas it abrogated the additive effect of APRIL in getting LLPCs with SCs. In

addition, a set of genes, whose expression is induced by NF-κB pathway activation in malignant PCs,[33] is poorly expressed in LLPCs generated with SCs *in vitro* compared to BMPCs as previously mentioned by Cocco *et al*.[24] Thus the activation of the NF-κB pathway is not mandatory to generate LLPCs *in vitro*, but could enhance it. The current data are in line with recent findings showing that a combination of APRIL, BAFF, IGF-1, SDF-1 and VEGF can support modestly the *in vitro* 14-day survival of human PCs harvested from the BM, whereas STAT3 activating cytokines, in particular IL-6, are critical.[40]

Using more complex culture conditions, including IL-6, IL-21, IFN-α and SC-CM, a recent study has shown human LLPCs can be generated *in vitro* also.[24] That the *in vitro* long-term survival of human PCs can be supported by 2 growth factors (IL-6 and APRIL) only is quite surprising regarding the current view of the complexity of the PC niche, comprising SCs and various hematopoietic cells (eosinophils, dendritic cells, megakaryocytes, neutrophils, basophils).[6,14,41] SCs are thought to serve as docking cells bringing close together PCs and hematopoietic cells,[14] but also producing soluble factors promoting PC survival, in particular IL-6 and galectin.[42,43] The ability of hematopoietic cells to sustain PC survival is due mainly by their ability to produce APRIL.[9,11]

The fact that soluble growth factors can replace niche cells for the generation and survival of LLPCs *in vitro* suggests it could be the case *in vivo* and questions about the regulation of LLPC count *in vivo*. It is generally assumed that the tiny PC count in the BM is regulated by the rarity of BM niche cells[5], new PCs being in competition with old ones for the availability of niche cells.[44] The current finding suggests the PC niche is mainly a liquid niche comprising a life-sustaining mixture and concentration of chemokines and growth factors, which is likely the case close to the docking SCs

attracting both PCs and hematopoietic cells.[14] When entering the BM, if a PC cannot migrate close to a SC, it will not encounter the life-sustaining concentrations of soluble factors and die. But in case of deregulated production of these cell communication signals such as in inflammatory conditions, one can expect many PCs may survive *in vivo*. This may explain the accumulation of LLPCs in the spleen of patients with primary immune thrombocytopenia treated with Rituximab anti-CD20 mAb, in association with a 2-fold increase in BAFF concentration in the spleen compared to Rituximab untreated patients.[34] Besides genomic abnormalities, this could also explain the progressive accumulation of premalignant PCs and then malignant PCs in patients with malignant PC disorders who display increased plasma concentrations of IL-6,[45] APRIL or BAFF.[46]

The current finding of a mandatory role of IL-6 to promote the survival of human LLPCs *in vitro* questions the role of IL-6 for LLPC maintenance. In mice, whereas IL-6 produced by SCs is mandatory to get the survival of BMPCs *in vitro*,[16,17] the survival of transferred LLPCs is not impaired in *IL-6*$^{-/-}$ mice unlike *APRIL*$^{-/-}$ ones.[14] This is likely due to bias in the murine or human models used. In particular, whereas LLPCs can not be transferred in *APRIL*$^{-/-}$ mice[14], Ag-specific PCs can be generated in *APRIL*$^{-/-}$ mice using repeated Ag boosts indicating additional factors can replace APRIL.[14] In *IL-6*$^{-/-}$ mice, a role of the other cytokines able to trigger gp130 IL-6 transducer chain and/or STAT3 activation and to supplement for a deficit in IL-6 induced signalling to support PC survival has not been evaluated. This is the case in humans since a recent study has emphasized that inhibition of STAT3 activation by small compounds can fully block the *in vitro* 14-day survival of human PCs harvested from the peripheral blood or BM of healthy individuals. STAT3 activation in these PCs could be driven by either IL-6, IL-10 or IL-21.[40]

These *in vitro* models to get human PCs likely introduce some bias, in particular by the method to activate B cells (through BCR, CD40, or TLR), the combination of cytokines used to generate prePBs, PBs, early PCs and then LLPCs, the origin of stromal cells and the culture conditions influencing PC metabolism (nutrients, glucose, O_2 concentration).[10,21,24,26] In the current model, we used a stromal cell line obtained from tonsils because it grow easily until confluence and at confluence, can survive for several months without proliferating but providing a continuous SC support. In initial experiments, similar data were obtained in term of phenotype and long-term survival with BM SCs. But it is of major interest to investigate further whether the use of SCs from different tissue origins could change the phenotype and gene expression profiling of LLPCs, in particular their proximity with BMPCs. For example, a progressive loss of IgA secreting PCs occurred in cultures with tonsil SCs or SC-CM likely due to the lack of a critical survival factor for IgA PC survival *in vitro*. Adding APRIL can revert this loss, increasing 3.4 fold the survival of IgA secreting PCs and this could eventually also occur with BM SCs. The ease of the current model to get PCs *in vitro* will make possible further identification of these possible biases.

All prePBs and PBs generated in this model express CD19 whereas Chaidos *et al*.[47] reported recently the existence of $CD19^-CD38^+CD138^-$ plasmablasts (called Pre-PCs) in healthy individuals, together with the known $CD19^+CD38^+CD138^-$ PBs and $CD19^+CD38^+CD138^+$ PCs.[22] The malignant counterpart of these Pre-PCs are found in patients with MM. Running a supervised analysis of gene expression profiling of malignant Pre-PCs and PCs, Chaidos *et al*. found enrichment of genes coding for epigenetic pathways.[47] The *in vitro* model we used likely failed to generate these $CD19^-CD38^+CD138^-$ since all prePBs generated at day 4, PBs at day 7 and PCs at

412 day 10 express CD19.[10,26] In addition, the epigenetic genes differentially expressed
413 between malignant Pre-PCs and PCs could not classify the current *in vitro* generated
414 PBs, early PCs and LLPCs (data not shown).
415 Besides its interest for understanding the fine pathways controlling PC generation
416 and survival in humans, the current model should be promising to study the
417 mechanisms involved in malignant PC disorders and controlling the activity of drugs
418 used to treat patients with these disorders. As several genes whose expression or
419 abnormalities are associated with disease activity have been identified,[48-55] their
420 modulation throughout the different stages of PC generation (prePBs, PBs, early
421 PCs, LLPCs) could help to understand better their function. Of note, it is now feasible
422 to force or repress the expression of a given gene in these PCs using measles
423 envelop pseudotyped lentiviral delivery.[56] The same holds true for drugs used to treat
424 patients with MM, in particular to identify if these drugs could target a specific PB or
425 PC stage and the underlying mechanism.

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AUTHOR CONTRIBUTIONS

MJ designed research, performed the experiments and wrote the paper.

KT, TF and FG, provided stromal cells and corrected the paper.

MC, NR, and KB performed the experiments.

CD provided assistance for cytometry experiments.

DH provided data of gene expression profiling of BMPCs.

BK is the senior investigator who designed research and wrote the paper.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

Supplementary information is available at Leukemia's website.

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FIGURE LEGENDS

Figure 1. Generation and survival of mature CD138^{bright} PCs *in vitro*.

(A) PCs (CD38^{+}CD138^{+} cells) were generated with or without stromal cells (SCs) in the presence of IL-6 (10 ng/ml) and cell count and viability assayed at day (D) 10, 14, 24, 30, and 60 using trypan blue dye exclusion. Data are the mean concentration of viable cells ± SD determined in six separate experiments. *The mean value is significantly higher than that in culture without SCs at the same culture day using a paired *t*-test ($P \leq .05$). (B) Expression of CD138. The histograms show FACS labelling with an anti-CD138 (black) or an isotype-matched control mAb (white) of one experiment representative of four. The percentages of CD138 positive cells and CD138 staining index (SI) are indicated in the panels. (C) CD138 fluorescence staining index. Data are the mean CD138 staining index ± SD of four separate experiments. Statistical analysis was done using a paired *t*-test. ns = not significant.

Figure 2. IL-6 in combination with APRIL or BAFF or SC-CM supports the generation and survival of PCs *in vitro*.

(A) D10 early PCs were FACS sorted and cultured from D10 to D30 either in contact with SCs (culture with SCs), or with SCs placed in a lower chamber of a transwell culture plate (SCs+PCs in transwells), or with supernatant of PC and SC coculture (PC/SC-CM), or culture supernatant of SCs (SC-CM). Results are the mean of viable cell counts ± SD of 3-paired experiments. ns: the mean count of PCs at a given culture day is not significantly different from that in the SC group at the same culture day using a paired *t*-test (B) FACS-sorted early PCs were cultured from D10 to D30 with culture medium and 10% FCS (Co) or in the presence of recombinant cytokines and/or the SC-CM. The concentrations of cytokines used were 10 ng/ml for IL-6 and IGF-1 and 200 ng/ml for APRIL, BAFF, and SDF-1. Counts of metabolic active cells

were assayed quantifying intracellular ATP amount with a Cell Titer Glo Luminescent Assay. Results are the mean ± SD of the luminescent signals expressed as the percentage of that in the group cultured with the SC-CM, determined in 3- to 7-paired experiments. *The mean value is significantly different from that in the SC-CM group using a paired *t*-test. **The mean value is significantly different from that in the IL-6 group using a paired *t*-test ($P \leq .05$). (C) Effect of various inhibitors of cytokines on the generation of plasma cells induced by the SC-CM. The anti-IL-6 mAb was used at a concentration of 10 µg/ml, the TACI-Fc at 10 µg/ml, the AMD3100 (SDF-1 inhibitor) at 10 µM and the IGF-1R inhibitor (NVP-AEW541) at 1 µM. Metabolic active cells were assayed quantifying intracellular ATP amount with a Cell Titer Glo Luminescent Assay. Results are the mean ± SD of the luminescent signals expressed as the percentage of that in the group cultured with the SC-CM, determined in 3- to 10-paired experiments. *The mean value is significantly different from that in the SC-CM group using a paired *t*-test.

Figure 3. A NF-κB inhibitor does not affect the generation of PCs by SCs but inhibited the additive effect of APRIL.

PCs were generated with SCs only or with SCs and 200 ng/ml recombinant APRIL. The IKK16 NF-κB inhibitor (1 or 3 µM) was added for 4 days in 2 culture groups and PCs counted at the end of the culture. For each experiment the PC count was expressed as the percentage of the PC count obtained with the control group of the same experiment (SCs or SCs + APRIL). Results are the mean percentages ± SD of five separate experiments. *The mean percentage is significantly decreased compared to that in the SCs + APRIL group using a paired *t*-test.

Figure 4. D30 PCs have a more mature phenotype than D10 early PCs.

675 PC phenotype was assessed by flow cytometry. Results are the mean percentage ±
676 SD of positive cells and the mean staining index ± SD determined in 4 to 6 separate
677 experiments. *The mean value is significantly different from that in D10 PCs using a
678 paired *t*-test ($P \leq .05$).

679 **Figure 5. D30 PCs do not cell cycle.**

680 The cell cycle was assessed using DAPI staining and quantification of cells in the S
681 phase using bromodeoxyuridine (BrdU) incorporation and labelling with an anti-BrdU
682 antibody. (A) Dot plots show a representative experiment out of three. The
683 percentages of cells in the G0/G1, S, and G2/M phases are indicated. (B) Results are
684 the mean percentage ± SD of cells in the S phase of three separate experiments.

685 **Figure 6. D30 and D60 PCs are functional PCs that produce Igs continuously.**

686 FACS-sorted D10 early PCs were cultured with SCs (A-F) or growth factors as
687 indicated (G). (A-C) Cytoplasmic (cy) Igs (IgG, IgA, and IgM) (A), cy-light chains (B),
688 and surface (s) Igs (C) were assessed by flow cytometry. Results are the mean
689 percentage ± SD of positive cells from 5 separate experiments. (D) IgG, IgA, and IgM
690 production was assessed by ELISA. Results are the mean ± SD of Ig production in
691 pg per cell and per day determined in 3 separate experiments. (E) The number of
692 IgG-, IgA-, and IgM-secreting cells was assessed by ELISPOT. Results are the mean
693 Ig-secreting cell number ± SD from 4 separate experiments. (F) ELISPOTs from a
694 representative experiment are shown. (G) FACS sorted D10 early PCs were cultured
695 with SCs or with a combination of cytokines and/or SC-CM as indicated. The number
696 of Ig-secreting cells was determined by ELISPOT at D30 of culture. Results are the
697 mean Ig-secreting cell number ± SD from 3 separate experiments. *The mean value
698 is significantly different from that in D10 PCs using a paired *t*-test.

Figure 7. Gene expression of transcription factors involved in PC differentiation.

Naive B cells (BCs), D10 early PCs, and D30 PCs were FACS sorted. Gene expression of *IRF4, PRDM1, XBP1u, XBP1s, PAX5,* and *BCL6* assayed by real-time RT-PCR. The mRNA level in the different cell populations was compared assigning the arbitrary value 1 to gene expression in BCs. Data are the mean value ± SD of gene expression determined in 5 separate experiments. The ratio *XBP1s/XBP1u* in D10 early PCs and D30 PCs is shown. *The mean value is significantly different from that in D10 PCs using a paired *t*-test ($P \leq .05$).

Figure 8. Unsupervised clustering of gene expression profile of purified D4 prePBs, D7 PBs, D10 early PCs, D30 PCs, and BMPCs.

D4 prePBs, D7 PBs, D10 early PCs, D30 PCs and BMPCs were profiled using Affymetrix U133 plus 2.0 microarray and an unsupervised hierarchical clustering was run with the 5000 probe sets with the highest variance (log transform, center genes and arrays, uncentered correlation and average linkage). The 5 populations are classified into 2 major clusters, a PC cluster comprising D10 early PCs, D30 PCs and BMPCs (r = 0.11) and a plasmablast cluster comprising prePBs and PBs (r = 0.20). The horizontal lines represent the normalized and centered expression of each of the 5000 genes in the samples and are depicted according to the color scale shown at the bottom (-1.5 to 1.5 on a log base 2 scale).

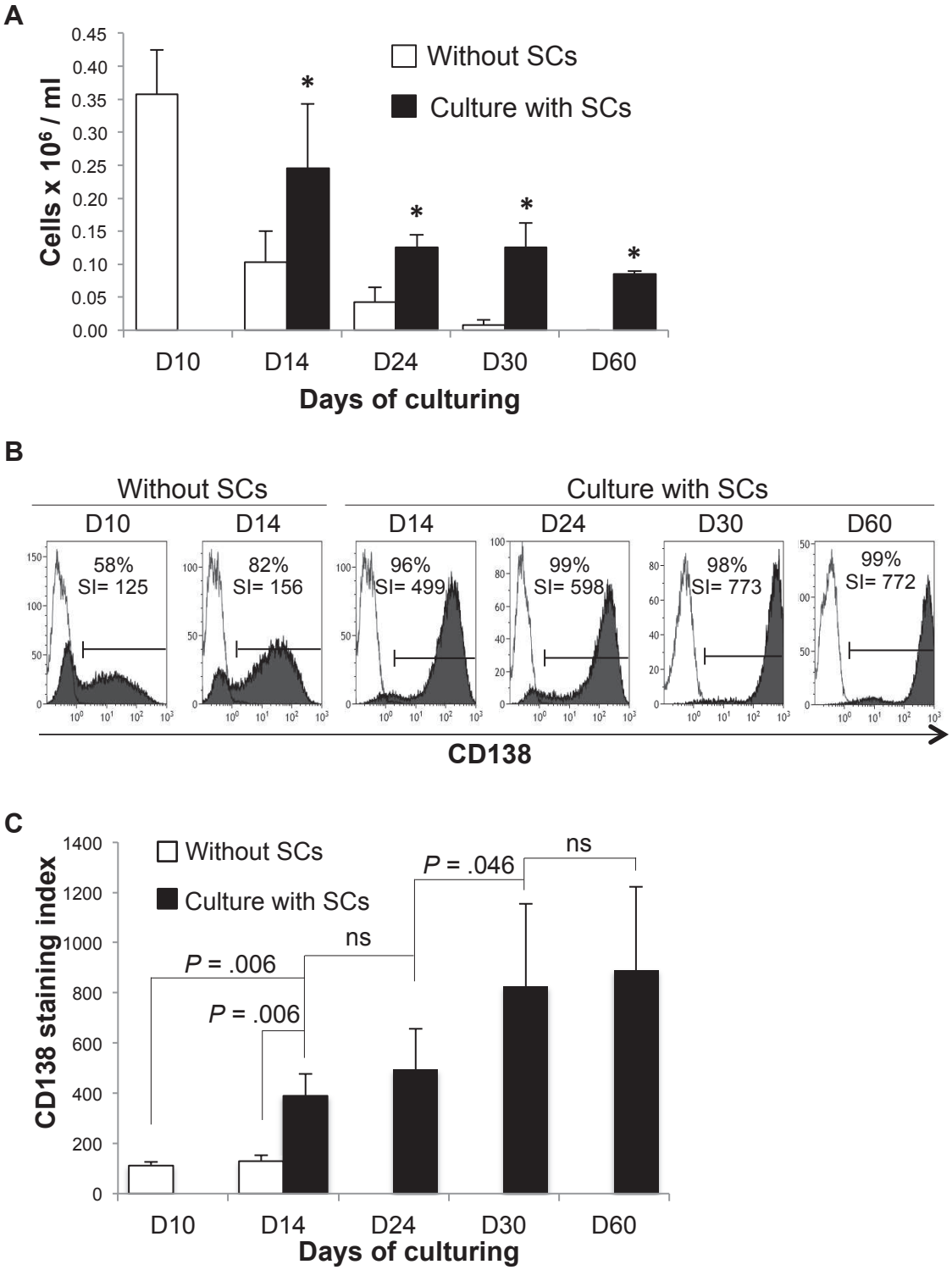
A
Cells x 10^6 / ml
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Without SCs
Culture with SCs
*
*
*
*
D10
D14
D24
D30
D60
Days of culturing
B
Without SCs
Culture with SCs
D10
D14
D14
D24
D30
D60
58%
SI= 125
82%
SI= 156
96%
SI= 499
99%
SI= 598
98%
SI= 773
99%
SI= 772
CD138
C
CD138 staining index
1400
1200
1000
800
600
400
200
0
Without SCs
Culture with SCs
P = .006
P = .006
ns
P = .046
ns
D10
D14
D24
D30
D60
Days of culturing

Figure 1

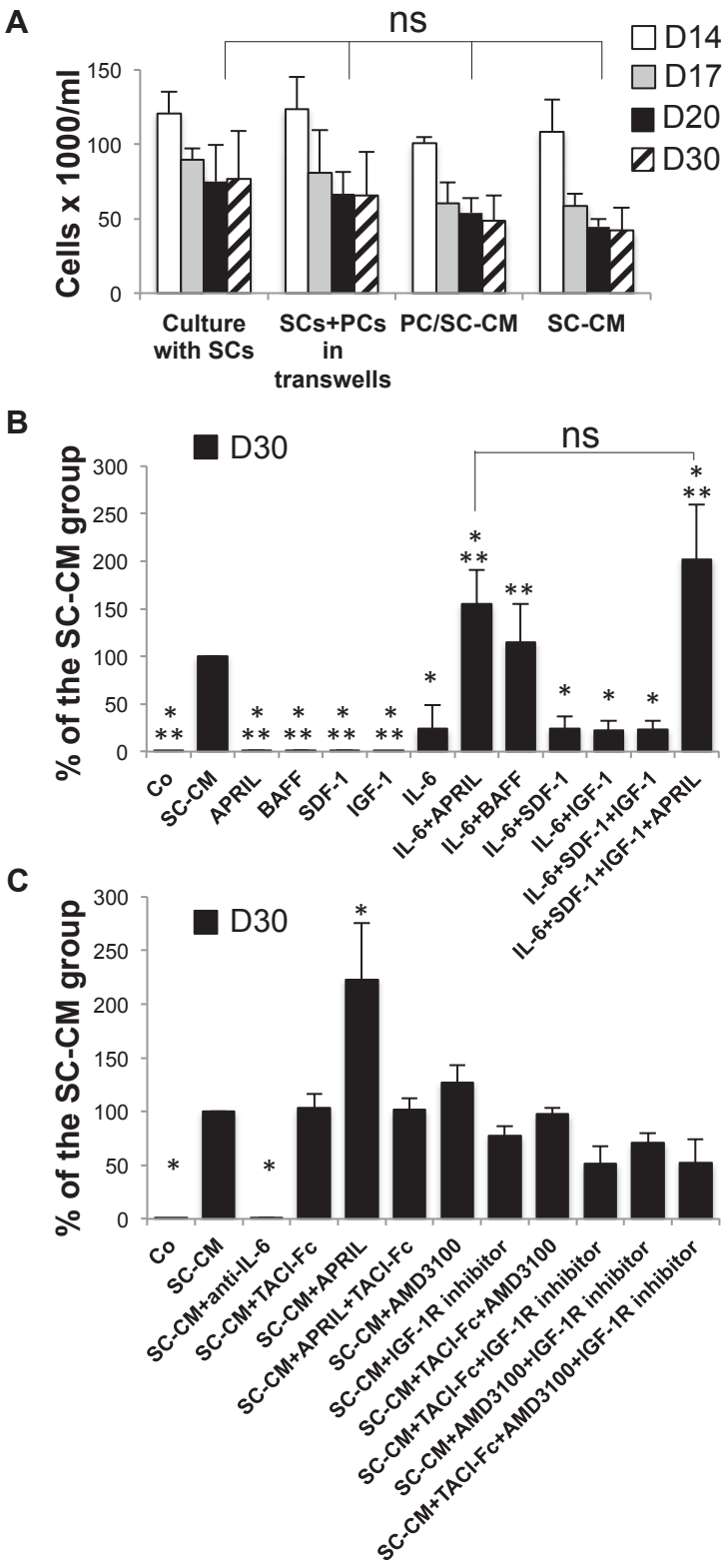
A
ns
D14
D17
D20
D30
Cells x 1000/ml
150
100
50
0
Culture with SCs
SCs+PCs in transwells
PC/SC-CM
SC-CM
B
D30
ns
% of the SC-CM group
300
250
200
150
100
50
0
Co
SC-CM
APRIL
BAFF
SDF-1
IGF-1
IL-6
IL-6+APRIL
IL-6+BAFF
IL-6+SDF-1
IL-6+IGF-1
IL-6+SDF-1+IGF-1
IL-6+SDF-1+IGF-1+APRIL
C
D30
% of the SC-CM group
300
250
200
150
100
50
0
Co
SC-CM
SC-CM+anti-IL-6
SC-CM+TACI-Fc
SC-CM+APRIL
SC-CM+APRIL+TACI-Fc
SC-CM+AMD3100
SC-CM+IGF-1R inhibitor
SC-CM+TACI-Fc+AMD3100
SC-CM+TACI-Fc+IGF-1R inhibitor
SC-CM+AMD3100+IGF-1R inhibitor
SC-CM+TACI-Fc+AMD3100+IGF-1R inhibitor

Figure 2

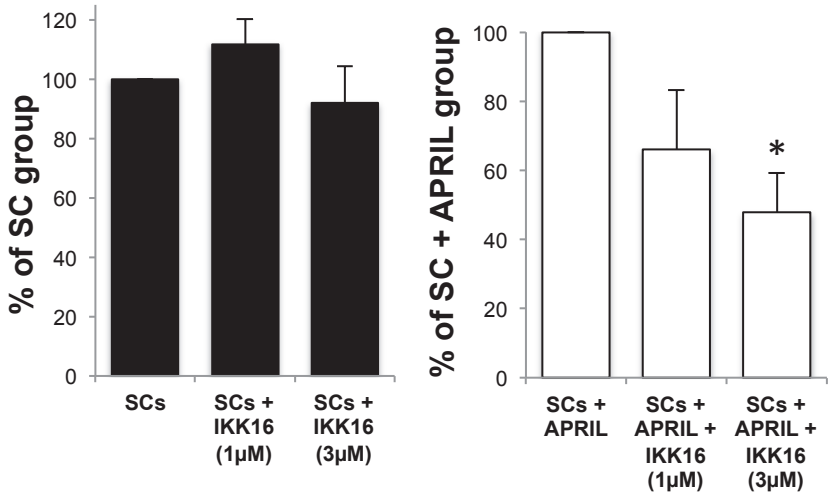
% of SC group
120
100
80
60
40
20
0
SCs
SCs + IKK16 (1μM)
SCs + IKK16 (3μM)
% of SC + APRIL group
100
80
60
40
20
0
*
SCs + APRIL
SCs + APRIL + IKK16 (1μM)
SCs + APRIL + IKK16 (3μM)

Figure 3

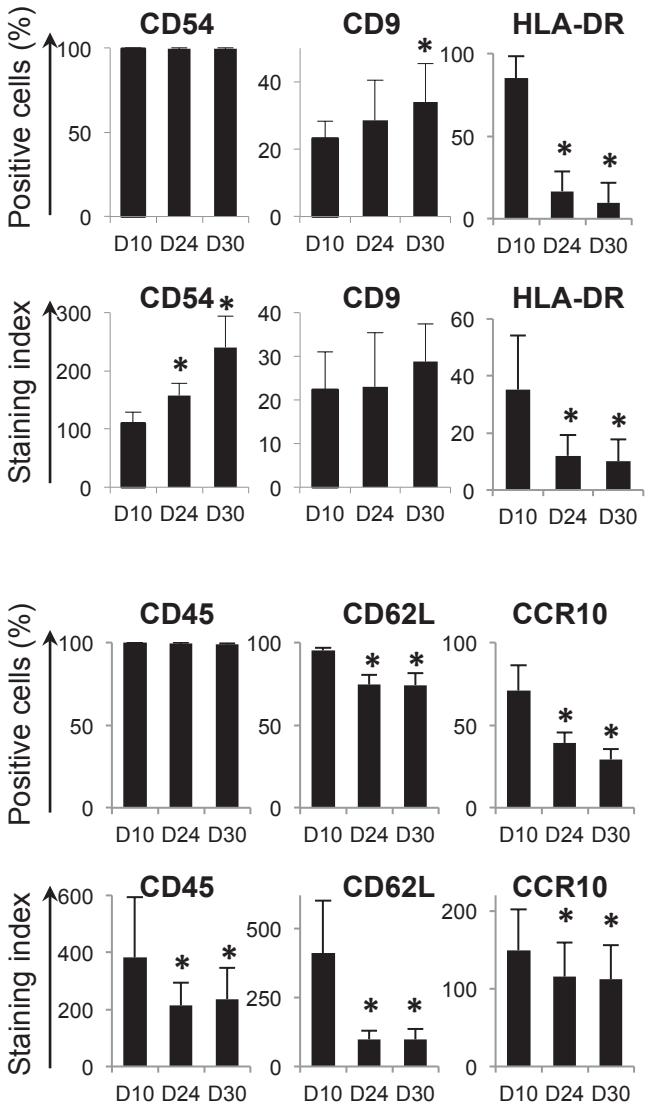
CD54
CD9
HLA-DR
Positive cells (%)
100
50
0
40
20
0
100
50
0
*
*
*
D10 D24 D30
D10 D24 D30
D10 D24 D30
CD54
CD9
HLA-DR
Staining index
300
200
100
0
40
30
20
10
0
60
40
20
0
*
*
*
*
*
D10 D24 D30
D10 D24 D30
D10 D24 D30
CD45
CD62L
CCR10
Positive cells (%)
100
50
0
100
50
0
100
50
0
*
*
*
*
*
D10 D24 D30
D10 D24 D30
D10 D24 D30
CD45
CD62L
CCR10
Staining index
600
400
200
0
500
250
0
200
100
0
*
*
*
*
*
*
*
D10 D24 D30
D10 D24 D30
D10 D24 D30

Figure 4

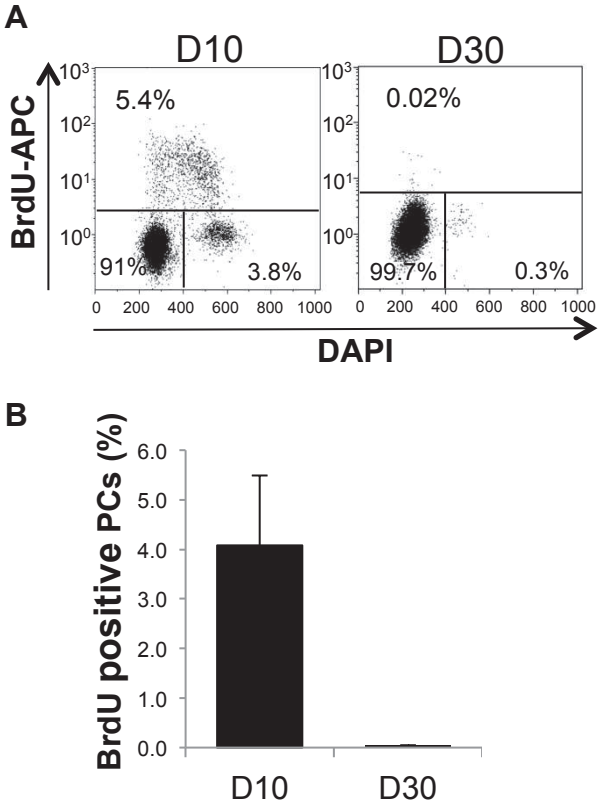
A
D10
D30
BrdU-APC
DAPI
5.4%
91%
3.8%
0.02%
99.7%
0.3%
B
BrdU positive PCs (%)
D10
D30

Figure 5

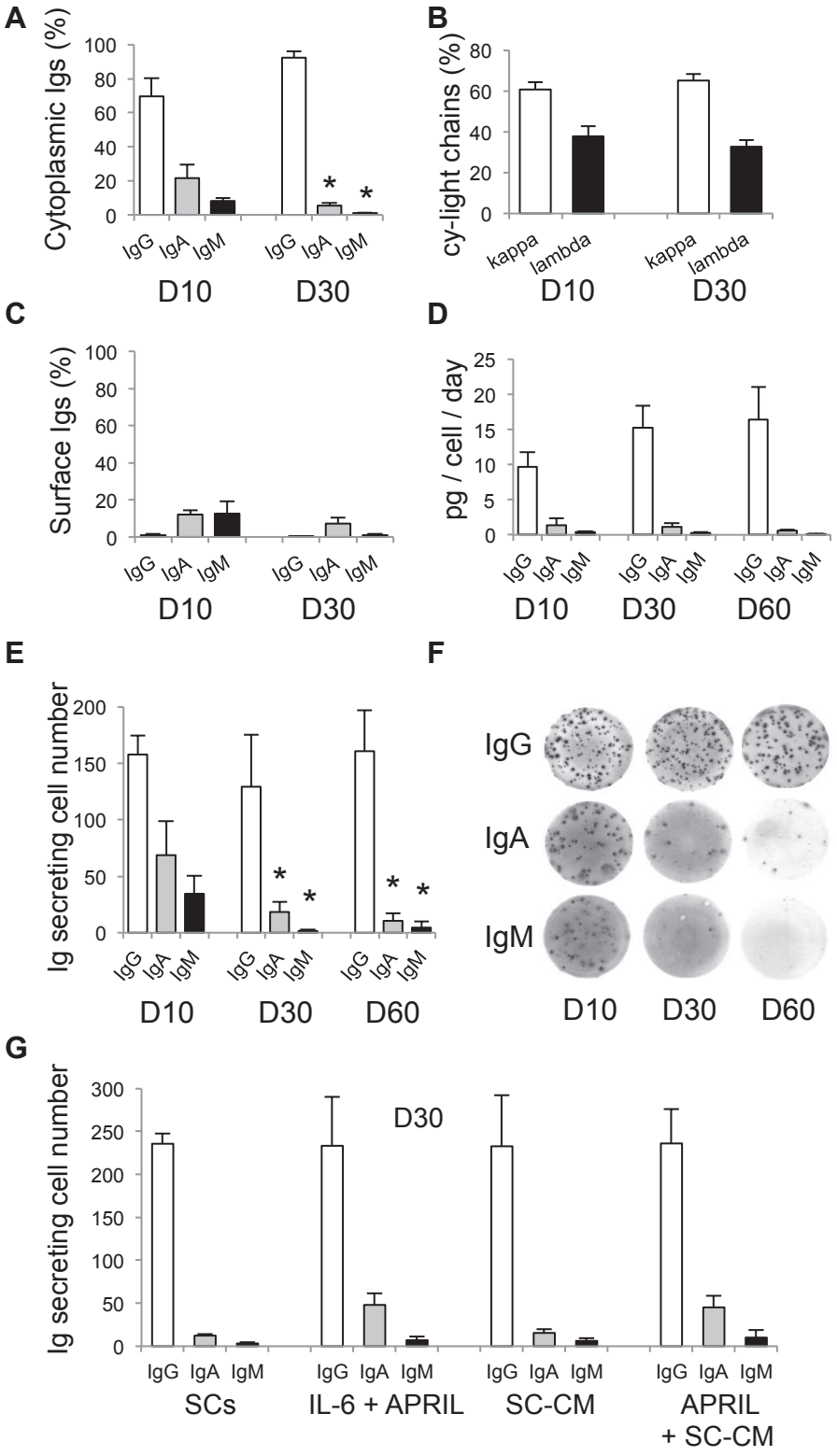
A
Cytoplasmic Igs (%)
100
80
60
40
20
0
IgG IgA IgM IgG IgA IgM
D10 D30
B
cy-light chains (%)
80
60
40
20
0
kappa lambda kappa lambda
D10 D30
C
Surface Igs (%)
100
80
60
40
20
0
IgG IgA IgM IgG IgA IgM
D10 D30
D
pg / cell / day
25
20
15
10
5
0
IgG IgA IgM IgG IgA IgM IgG IgA IgM
D10 D30 D60
E
Ig secreting cell number
200
150
100
50
0
IgG IgA IgM IgG IgA IgM IgG IgA IgM
D10 D30 D60
F
IgG
IgA
IgM
D10 D30 D60
G
Ig secreting cell number
300
250
200
150
100
50
0
D30
IgG IgA IgM IgG IgA IgM IgG IgA IgM IgG IgA IgM
SCs IL-6 + APRIL SC-CM APRIL + SC-CM

Figure 6

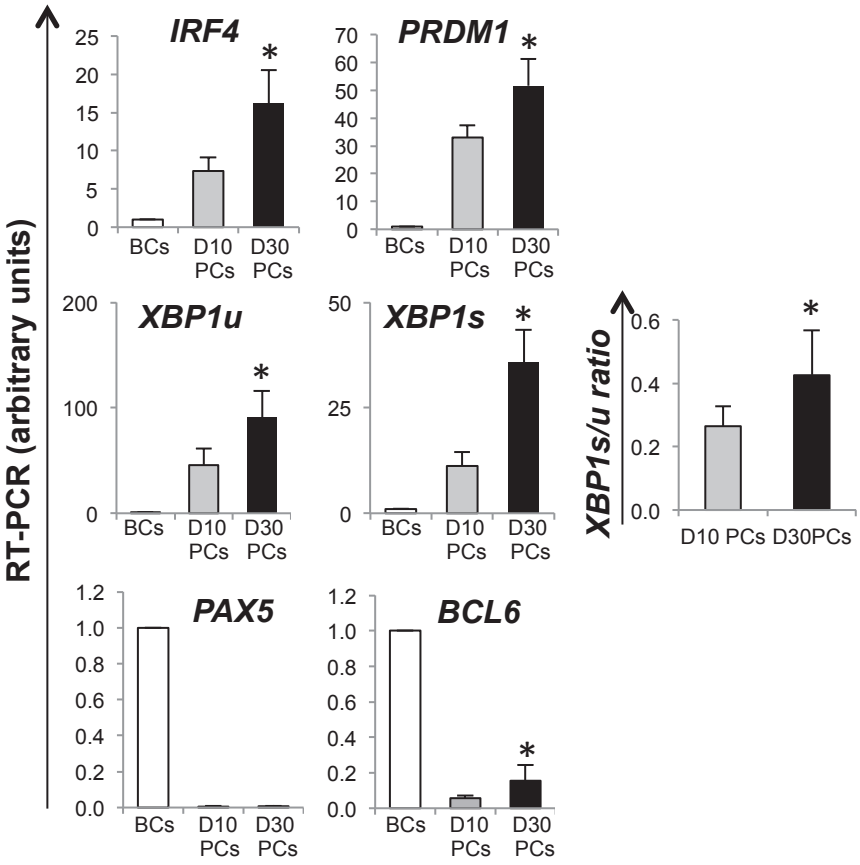
RT-PCR (arbitrary units)
IRF4
PRDM1
XBP1u
XBP1s
XBP1s/u ratio
PAX5
BCL6
BCs
D10 PCs
D30 PCs
D30PCs
*

Figure 7

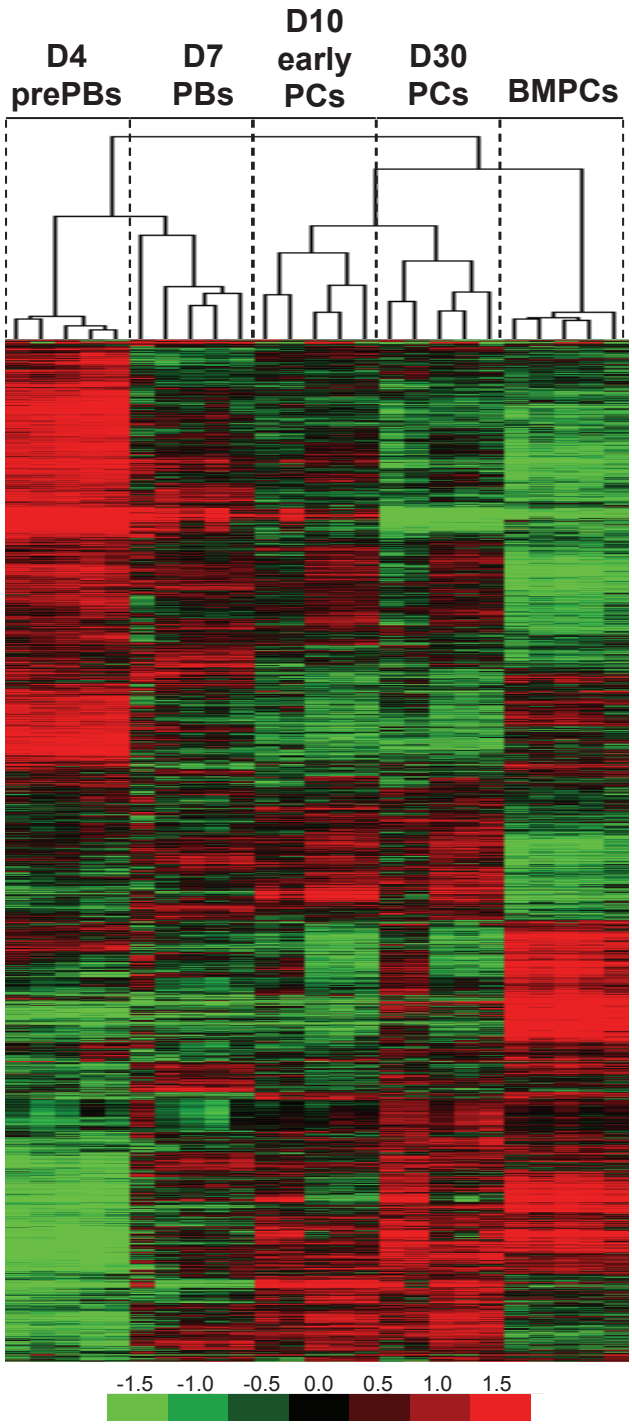

Figure 8